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Chemical Mediation of Larval Release Behaviors in the Crab *Neopanope sayi*

M. C. DE VRIES, D. RITTSCHOF, AND R. B. FORWARD JR.

Duke University Marine Laboratory, Beaufort, North Carolina 28516 and Duke University Zoology Department, Durham, North Carolina 27706

Abstract. Control of egg hatching was investigated in ovigerous females of the crab *Neopanope sayi*. Larval release is a brief event, generally lasting less than 15 min, during which females perform stereotypic behaviors involving vigorous abdomen pumping. Substances released by hatching eggs (pumping factors) of *N. sayi*, *Rhithropanopeus harrisii*, and *Uca pugilator*, but not *Sesarma cinereum*, evoked these stereotypic behaviors (pumping response) in ovigerous *N. sayi*. Spontaneous pumping and responsiveness to pumping factors varied with the age of the embryos. These results indicate that the eggs release pheromones around the time of hatching, which supports the general model for egg-hatching control described for *R. harrisii* (Forward and Lohmann, 1983). The chemistry of *N. sayi* pumping factors was investigated, and the pumping response was used as a bioassay in this study. Pumping factors adsorbed to Amberlite XAD-7 resin and could be eluted from it with methanol. Size fractionation by cascade pressure dialysis showed that the active molecules were <1000 daltons. Acid hydrolysis followed by reverse-phase HPLC amino acid analysis showed that the biologically active fraction contained peptides. Cysteine, glycine, methionine, and isoleucine were the four most common amino acids in these peptides. The responsiveness of *N. sayi* to hatch water from *R. harrisii*, the general similarity of adsorptive characteristics of hatch waters from the two species toward XAD-7 resin, and the amino acid compositional analysis suggest that the pumping factors from both species are similar. This supports the hypothesis that *N. sayi* pumping factors are also small peptides, as was suggested for those of *R. harrisii* (Rittschof *et al.*, 1985, 1989).

Introduction

Rhythms in larval release corresponding to lunar, diel, and tidal cycles have been observed for numerous species of Brachyura (see DeCoursey, 1983; Forward, 1987, for reviews). In species showing rhythms, egg hatching generally occurs during the dark phase of the diel cycle, and often near the high tide of a tidal cycle (Saigusa and Hidaka, 1978; Saigusa, 1981, 1982; Forward *et al.*, 1982; Wolcott and Wolcott, 1982; Christy, 1986; Salmon *et al.*, 1986; De Vries and Forward, 1989). For most warm-water species, such as the xanthid *Neopanope sayi*, larval release is a brief event, usually lasting less than 15 min for an individual (DeCoursey, 1979; Forward *et al.*, 1982; De Vries, 1990). During this time, in general, all of a female's eggs hatch while the female vigorously pumps her abdomen. Occasionally, larvae are released in more than one short burst at the time of consecutive tidal phases or nights (Forward *et al.*, 1982; Christy, 1986; De Vries and Forward, 1989).

The control of egg-hatching time in decapods seems to vary with species. Hatching time has been reported to be controlled by the female (Branford, 1978; DeCoursey, 1979) and alternatively by the developing embryos (Pandian, 1970; Ennis, 1973; Forward and Lohmann, 1983) in crabs and lobsters. The site of egg-hatching control may also be related to adult habitat (De Vries and Forward, 1991a). Control of egg-hatching time has been well studied only in the subtidal xanthid crab *Rhithropanopeus harrisii* (Forward and Lohmann, 1983; Rittschof *et al.*, 1985; Forward *et al.*, 1987; Rittschof *et al.*, 1989). In this species, the developing embryos control the exact timing, while the female controls the synchrony of hatching. Substances associated with hatching eggs are released near the time of hatching and induce the ovigerous female to perform stereotyped larval release behaviors that synchronize hatching. These pheromones are collectively called "pumping factors" and are a heterogeneous group of peptides mostly of <500 daltons.

The present study was performed to investigate the generality of the model for hatching-time control de-

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scribed for *R. harrisii* (Forward and Lohmann, 1983). In particular, we aimed to determine whether active substances from another xanthid, *Neopanope sayi,* are similar to active components from *R. harrisii.* If active molecules from *N. sayi* are similar to those from *R. harrisii,* they should cross react biologically, and produce similar results upon chemical purification and analysis, as done for *R. harrisii* pumping factors (Rittschof *et al.,* 1985).

Neopanope sayi is a subtidal xanthid crab, occurring in coastal and estuarine areas, from the low littoral to the sublittoral zones (Williams, 1984). Experiments were designed to determine whether hatching eggs of *N. sayi* and other crab species produce substances that stimulate ovigerous *N. sayi* to perform larval release behaviors. Pumping factors were indicated, and some of their chemical characteristics were investigated, the crabs' stereotyped larval release behavior serving as an assay for biological activity. Our results suggest that pumping factors from *N. sayi* are similar in composition, but not identical, to those from *R. harrisii.*

Materials and Methods

General collection and maintenance of animals

Ovigerous females of *Neopanope sayi* (Smith) were collected from among the subtidal hard substrate community near the Duke University Marine Laboratory in Beaufort, North Carolina. Crabs were brought into the laboratory and placed into individually numbered culture bowls (diameter, 10.4 cm) containing approximately 160 ml of 5 μm filtered ambient salinity seawater (approximately 32–35‰). Crabs were located in a controlled-environment room (27°C ± 1°C), under a 14 h light:10 h dark cycle, with lights-out at 2000 h. This LD cycle corresponded to the cycle in the field at the time of collection.

The water in each crab's bowl was changed daily between 0900 and 1200 h. At this time, the presence of larvae in the bowls was noted and the date of larval release recorded for each crab. Experiments were performed on ovigerous females and the data were examined in relation to the age of the embryo. Embryonic age at the time of experimentation (expressed as days until hatching) was determined by counting backwards from the subsequent time of hatching. For individuals that released larvae in more than one burst, the release date was considered to be on the day that the first group of eggs hatched. Crabs were not fed while in the laboratory.

Spontaneous levels of pumping

Until they released their larvae, the crabs were placed once each day for 2 min into filtered (0.45 μm) seawater, and the number of spontaneous pumps counted. The spontaneous pumping activities of 62 *N. sayi* carrying embryos of various developmental stages were recorded in this way. These data were collected to determine whether the percentage of crabs that pumped spontaneously, and the absolute frequency of spontaneous pumping varied with embryo age.

Because crabs were brought into the laboratory carrying embryos of all ages, and because their pumping activities were measured repeatedly, the effects on spontaneous pumping activity of length of time in the laboratory and of embryo age might be confounded. To separate the effects of the two variables, plots of frequency of spontaneous abdomen pumping *versus* length of time in the laboratory were made for three groups of crabs (*i.e.,* those carrying embryos that would hatch in 0–1, 2–3, and 4–5 days, respectively). These plots showed no relationship between pumping and time in the laboratory (De Vries, unpub. data). Thus, when embryo age was held constant, the frequency of spontaneous abdomen pumping appeared to be independent of time in the laboratory. Plots were not made for crabs carrying embryos in earlier stages because of the small numbers and extremely low pumping rate of such crabs. *N. sayi* release their larvae within at most 10 days of egg deposition at the laboratory maintenance temperature of 27°C.

Biological assays

An abdomen-pumping bioassay was used to detect chemicals that stimulated larval release behaviors in ovigerous crabs. The assay, a modification of that described in Forward and Lohmann (1983), proceeded as follows. A crab was placed in a bowl (diameter, 7.9 cm) containing 80 ml of filtered, (0.45 μm) ambient salinity seawater, and the frequency of abdomen pumps was counted for 2 min. The crab was then transferred to a second bowl containing 80 ml of test solution, and the count was repeated. If a crab pumped at least five more times in the second bowl than in the first, this was counted as a positive signal or a "response" to that test solution. For any given test solution, 20–60 (but usually 30) animals were assayed. The percentage of crabs tested that responded to each test solution was defined as the % response, and is considered a measure of the biological activity of that solution. Most test solutions were derived from water in which crab larvae were released by females. We used the number of larvae released per ml of water as an indication of the concentration of active substances in that solution. The results of pumping-response assays are shown as dose-response curves (*i.e.,* larvae/ml *vs.* % response), such as that in Figure 2.

The two-bowl protocol, described above, was used to allow for variability in spontaneous pumping activity among individuals, as well as for changes in this parameter within an individual that might occur between 1000 and 1700 h, the interval during which these assays were performed. An assay was also performed in which both bowls

contained filtered seawater—a control of the effects of the experimental procedure upon spontaneous pumping rates. Although pumps were usually vigorous, they were sometimes subtle, and could be unseen if crabs suddenly moved their abdomens out of view. The criterion of a five pump difference to define a positive response was therefore used to preclude potential observational errors that could occur with differences of less than five pumps. A simple proportional increase in pumping between two bowls to define a response was inappropriate, because many crabs pumped 0 times in the first bowl.

Water in the control and test bowls was replaced after every 10 crabs to ensure a minimal change in water composition between crabs (Forward *et al.*, 1987). Individuals were assayed in each concentration of a test solution only once, and were not retested with another concentration within 30 min. Individuals were generally tested 3–5 times/day. Crabs were returned to their home bowls containing filtered seawater between tests. Substances were tested from the lowest to the highest concentrations to reduce adaptation. Significant differences between test and control response levels were established by the use of a Z-statistic for testing differences between two proportions at $\alpha = 0.05$ (Walpole, 1974).

Preparation of hatch water

We collected water into which *N. sayi* had released larvae and determined whether it would stimulate larval release behaviors (*i.e.*, abdominal pumping) in ovigerous crabs. About 1–2 h before the predicted time of larval release (generally evening high tide for *N. sayi;* De Vries and Forward, 1989), ovigerous crabs were placed into individual culture bowls. The bowls were 10.4 or 7.9 cm in diameter (depending upon crab size) and contained 100 or 50 ml (respectively) of 0.45 μm filtered ambient salinity seawater. Just after a female had released her larvae, she was removed from the bowl, and the water was passed through 100 μm plankton netting to remove the larvae. This filtered hatch water was kept on ice only briefly and was then frozen (−20°C) for later use. The titer of pumping factors in a hatch water sample was estimated by counting subsamples of the larvae contributing to it, and is expressed as larvae/ml. Hatch water was collected from three additional crab species, *Sesarma cinereum* (Grapsidae), *Uca pugilator* (Ocypodidae), and *Rhithropanopeus harrisii* (Xanthidae), as described above, except that *R. harrisii* hatch water was collected in 10‰ water, as necessitated by their upper estuarine habitat. For testing in biological assays with *N. sayi*, the hatch water from *R. harrisii* was raised to ambient salinity with Instant Ocean.

The response to hatch water from *N. sayi* was assayed with crabs carrying embryos of different ages to determine whether sensitivity changed with the stage of embryonic development. Crabs with early (>5 days until hatching) embryos and crabs with late-stage (≤3 days until hatching) embryos were tested, and control levels (*i.e.*, percent response) were established for them. For all other assays, however, only crabs with late-stage embryos were used.

Isolation and purification of pumping factors

Adsorption chromatography. The molecular characteristics of *N. sayi* pumping factors were determined by a modification of the adsorption chromatography and size fractionation procedures of Rittschof *et al.* (1985), with the pumping bioassay being used to monitor the process. The pumping factors from hatch water were first concentrated on Amberlite XAD-7 resin. The column of resin (24 cm × 1 cm bed volume) was stored in 100% HPLC-grade methanol (Fisher Chemical Co.), and immediately before being loaded with pumping factors was rinsed with hexane, then back flushed and rinsed with at least 200 ml of deionized water. Loading was done by gravity-feed at approximately 16 ml/min. The passage of hatch water through the column was stopped before the resin was exposed. Methanol was carefully overlaid upon the hatch water. At the first signs of methanol breakthrough in the eluate (decrease in drop size and effervescence), the next 13 ml of solution was collected. The methanol in this sample was then evaporated under a stream of N_2 until approximately 1–2 ml of solution remained. This concentrated pumping factor was stored at −20°C until it was bioassayed or size fractionated.

For bioassays, concentrated pumping factor was diluted with filtered (0.45 μm) seawater to the desired test concentration. The calculation of larval concentrations in the test solutions was based upon that estimated in the original hatch water samples, assuming 100% recovery of pumping factors from the resin.

Cascade pressure dialysis. Hatch water from *N. sayi* that had been concentrated on XAD-7 resin was brought to a volume of about 100 ml with deionized water. This solution was subjected to cascade pressure dialysis (4°C, 40 psi); Amicon YM10 and YM2 Diaflo membranes with nominal cutoffs at 10 and 1 kDa, respectively, were used. The membranes were stored and rinsed according to the manufacturer's instructions. Two additional rinses with 50 ml deionized water were carried out under pressure to insure that all preservatives were washed from the membranes. A sample was first passed through the 10 kDa cutoff membrane. Part of this filtrate was bioassayed, and the remainder was fractionated with the 1 kDa cutoff membrane. When about 10 ml of sample remained above each membrane, three successive 40 ml rinses with distilled water were done. This procedure effectively eliminated small molecules (<10 or <1 kDa) in the original solution that might have been passively retained above the membranes. Rinse water passing through the membranes was discarded. Those solutions that passed through

the membranes, as well as those that were retained, were kept on ice and bioassayed immediately.

For bioassays, size fractionated pumping factor was diluted with filtered (0.45 μm) seawater to the desired test concentration. Larval concentrations in the test solutions were calculated based on those estimated in the original hatch water sample, assuming 100% recovery of pumping factors from size fractionation procedures.

Control solutions

To be certain that substances with biological activity were directly related to the hatching process, two control solutions were subjected to the hatch water purification procedure described above. One solution was 0.45 μm filtered seawater. The other was seawater in which ovigerous crabs had been incubated under conditions similar to those used for crabs releasing larvae (ovigerous crab essence). The filtered seawater was processed to ensure that no stimulatory effects were produced by the adsorption chromatography or cascade pressure dialysis. A volume of seawater equal to the average volume of hatch water processed in each batch was used. The number of larval equivalents in the filtered seawater control was based on the average concentration of all *N. sayi* hatch water processed during the present experiments.

Ovigerous crab essence was included as a control to ensure that substances associated with hatching eggs, and not substances secreted by ovigerous crabs or their embryos at other times, were responsible for the observed pumping activity. Ovigerous crab essence was prepared by placing 20 ovigerous *N. sayi* with embryos of various developmental stages into 2 l of 0.45 μm filtered seawater. After 2 h, the crabs were removed and the water treated as described above for the filtered seawater control. Each crab was assumed to carry 2500 eggs (based on unpublished estimates of egg-mass sizes for *N. sayi*), from which the larval concentrations of this control were calculated. Aliquots of the seawater and ovigerous crab essence controls were diluted such that they contained concentrations equivalent to 20 and 50 larvae/ml and were assayed; these concentrations of crude and size-fractionated hatch water produced strong pumping responses.

To be certain that larger active molecules were not denatured on the column matrix upon elution, hatch water not passed through the column was filtered through a 10 kDa membrane. Dilution series of the <10 kDa and >10 kDa fractions were assayed.

Amino acid analysis

The amino acid composition of *N. sayi* pumping factors was analyzed by Dr. Dano Fiorio at Florida State University. A <1 kDa sample from the release of approximately 19,000 larvae was analyzed. Reverse-phase high-performance liquid chromatography (HPLC) and pre-column derivatization with phenylisothiocyanate were performed using a modification of the method in Henrickson and Meredith (1984). Unhydrolyzed and hydrolyzed (in 6 *N* HCl for 24 h at 110°C) samples were analyzed to determine the initial composition of free amino acids and the composition of the peptides (<1 kDa), respectively. Phenylthiocarbomyl derivatives were separated on an octadecasilyl reverse-phase column and detected spectrophotometrically at 254 nm. Identification and quantification of amino acids was by comparison of derivatized standard amino acids with those in the samples.

Amino acid experiments

Results of the above compositional analysis provided the basis for testing the biological activity of mixtures of pure amino acids (Sigma Chemicals). Pumping assays were performed using a mixture of the four most abundant amino acids in hydrolyzed pumping factor, L-cysteine, glycine, L-isoleucine, and L-methionine. These amino acids were combined in equimolar amounts (as in hydrolyzed factor) and tested at concentrations bracketing those for which hatch water was active. In addition, pumping assays were performed using a combination of glycine and arginine, the most abundant amino acids in *Rhithropanopeus harrisii* pumping factor, in proportions and concentrations bracketing their level in hydrolyzed pumping factor from this species (Rittschof *et al.*, 1985).

Results

Spontaneous pumping rates

Frequency of spontaneous abdomen pumping generally increased with the age of the embryos (Fig. 1A). For crabs with embryos of all ages, at least 25% did not pump at all. However, the percentage of crabs which pumped increased sharply with increasing embryo age (Fig. 1B).

Response to hatch water

The percent pumping response of ovigerous *N. sayi* individuals increased when the animals were exposed to hatch water (Fig. 2A). Responsiveness varied with concentration for crabs with both early- and late-stage embryos. At concentrations lower than 2.5 larvae/ml for crabs with late embryos, and lower than 5.0 larvae/ml for crabs with early embryos, the percentages of crabs responding were not significantly different from controls. At these concentrations and higher, however, the percentages of crabs responding were significantly greater than controls.

For each concentration tested, the percentages of crabs with early embryos that responded were consistently lower than those of crabs with late embryos. This reflects, in part, the greater inclination of crabs with older embryos to spontaneously pump their abdomens (as evidenced by

the increased control levels), but probably also indicates that crabs with older embryos are more sensitive or responsive to pumping factors. The latter is evidenced by the higher concentration of hatch water necessary to elicit a significant percent response for crabs with early embryos compared to those with late embryos. Because crabs with late stage embryos were more responsive to pumping factors, they were used in all subsequent experiments.

N. sayi with late embryos also had significantly higher percent pumping responses upon exposure to hatch water from *Rhithropanopeus harrisii* and *Uca pugilator* at concentrations ≥ 20 larvae/ml (Fig. 3). Exposure of *N. sayi* to hatch water from *Sesarma cinereum* at concentrations from 1 to 60 larvae/ml, however, produced levels of response not significantly different from the control (Fig. 3). These results indicate some, but not complete, cross-reactivity of hatch waters among species, and suggest that active substances from some species are similar in composition.

Adsorption chromatography and cascade pressure dialysis

Hatch water was fractionated into substances with and without affinity for Amberlite XAD-7 resin, and the fractions were bioassayed with crabs bearing late-stage embryos. A concentration of 10 larvae/ml was tested because it produced maximum response (Fig. 2A). When exposed to untreated hatch water, 69% of the crabs responded (Table I), which was significantly greater than the control level (23%; n = 158). After passage of hatch water through the resin however, activity was lost. Only 33% of the crabs responded, which was not significantly different from the control level.

To recover adsorbed activity, the resin was eluted with methanol, which removes lipophilic and proteinaceous substances. Bioassays showed a modest increase in response when tested with the methanol eluate, but at concentrations up to 20 larvae/ml, these responses were not significantly different from those of the control (Fig. 2B, before size fractionation). After passage of the methanol eluate through the 10 kDa and 1 kDa membranes, activity reappeared (Fig. 2B), presumably due to the removal of an inhibitor introduced by, or concentrated by, the resin (see Discussion). In these two fractions, an increase in the percentage of response with concentration was observed, with concentrations ≥ 10 larvae/ml producing levels of response significantly different from control. The fractions from these two filtrations, which were retained above the membranes (the >10 kDa and the <10 kDa but >1 kDa fractions), produced levels of response no different from controls at concentrations up to 20 larvae/ml.

When untreated hatch water was passed through the 10 kDa cutoff membrane, the retained >10 kDa fraction lacked biological activity (Fig. 4). Response levels in the

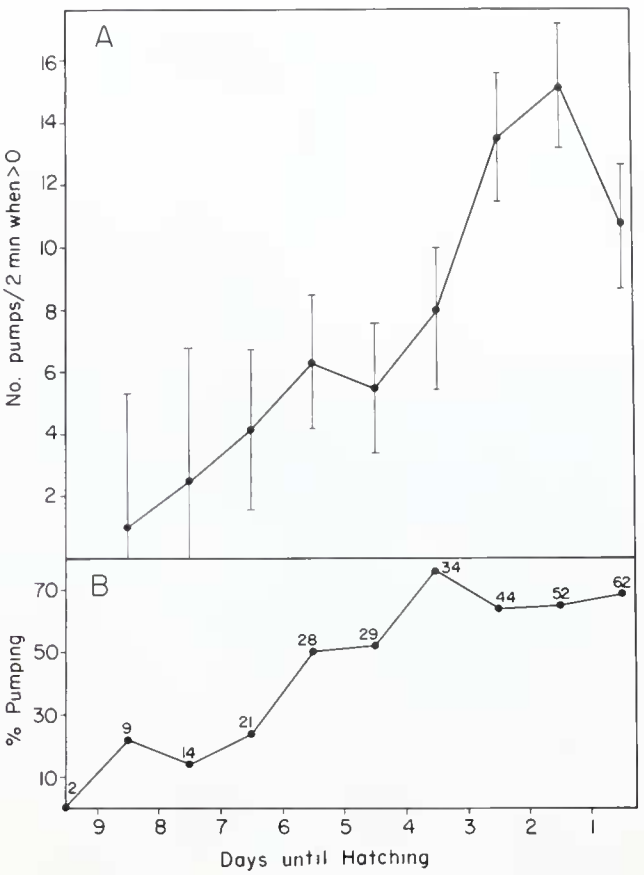

Figure 1. Frequency of spontaneous abdomen pumping (A), and percentage of crabs which pumped (B), as a function of embryo age in ovigerous female *Neopanope sayi*. Means and 95% confidence limits of spontaneous pumping frequency are shown for crabs that pumped at least once. The numbers beside the points are the sample sizes.

<10 kDa fraction were significantly greater than control levels at ≥10 larvae/ml. The absence of biological activity in the >10 kDa fraction of the untreated hatch water suggests that precipitation of large (>10 kDa), biologically active molecules onto the resin upon methanol elution was unimportant. In summary, pumping factors were adsorbed to XAD-7 resin, eluted with methanol, and were <1 kDa.

Controls demonstrated that biologically active substances originate from hatching eggs. Filtered seawater and ovigerous crab essence were subjected to the adsorption chromatograph and fractionation procedures. At concentrations equivalent to 20 and 50 larvae/ml, neither filtered seawater nor crab essence produced responses significantly greater than the filtered seawater control, either before or after passage of the two solutions through the resin and membranes (Table II). These results show that passage of seawater through the resin and membranes did not add excitatory substances to the water, and that substances associated with ovigerous females, in the absence of egg hatching, did not produce a significant level of pumping response.

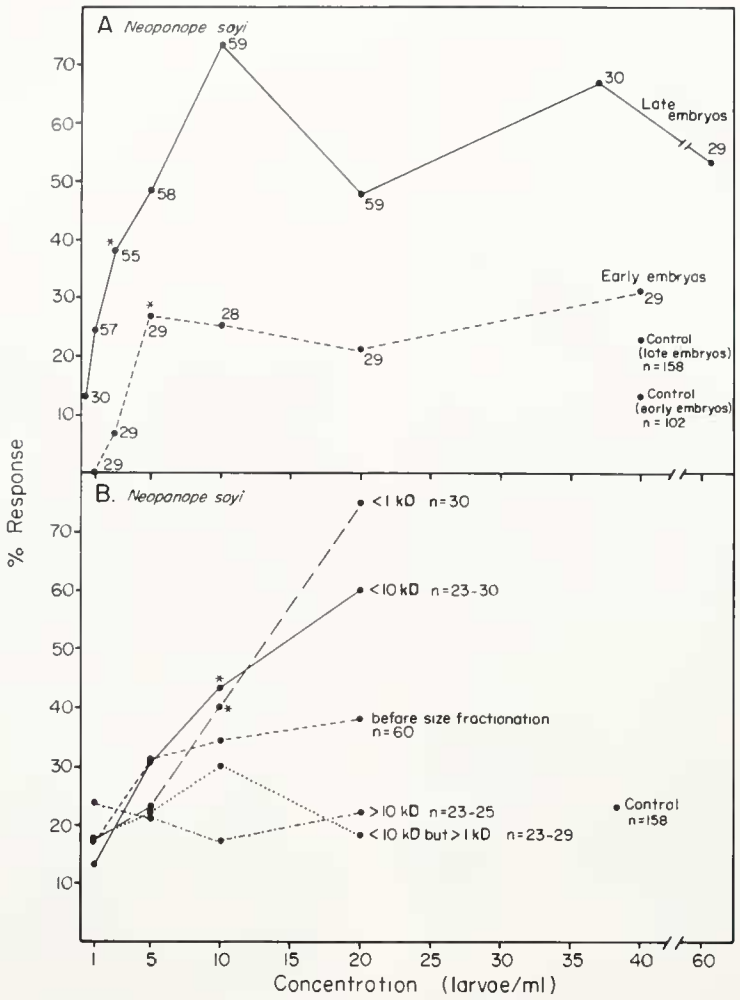

Figure 2. Percentage of ovigerous female *Neopanope sayi* that responded to *N. sayi* hatch water (A) and to various size fractions of a methanol eluate of hatch water after concentration on XAD-7 resin (B). Concentration of active substances (abscissa) was calculated from estimates of larval concentration in the untreated hatch water solutions. Numbers by points are specific sample sizes (A) or a range of sample sizes (B). Asterisks indicate the first concentration at which pumping response was significantly different from controls. Control levels for pumping response were established in filtered seawater. Crabs with late embryos were 0–3 days from larval release, and those with early embryos were >5 days from larval release.

Amino acid analysis

Reverse-phase HPLC analysis of free and hydrolyzable amino acids showed picomolar concentrations of 15 amino acids in the biologically active (<1 kDa) fraction of hatch water concentrated on the resin [calculated to a titer of 160 larvae/ml for comparison with results in Rittschof *et al.* (1985); Table III]. The most abundant amino acids after hydrolysis—cysteine, glycine, isoleucine, and methionine—accounted for 47% of the total free, and 57% of the total hydrolyzable amino acids. These four amino acids were approximately equimolar, at >100 p*M* after acid hydrolysis. Free amino acids before hydrolysis represented 23% of the total amino acids in the sample after hydrolysis. *N. sayi* pumping factors of this titer thus contain picomolar amounts of amino acids, most of which appear to be bound in peptides.

Amino acid experiments

When presented with mixtures of the amino acids most abundant in partially purified hatch water from *N. sayi* and *R. harrisii*, ovigerous *N. sayi* individuals significantly increased their levels of pumping over those of controls, at concentrations > 10^{-4} *M* (Fig. 5). Concentrations at which native pumping factors were effective (10^{-7}–10^{-9} *M*), produced pumping responses no different from controls. These effective concentrations are based on those of the four major amino acids (Table III) in hatch water of titer > 2.5 larvae/ml (*i.e.*, the threshold for crabs carrying late embryos; Fig. 2). In contrast, amino acid mix-

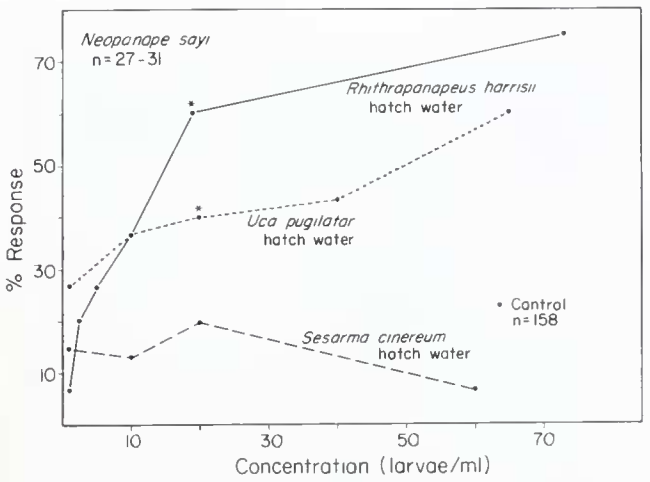

Figure 3. Percentage of ovigerous female *Neopanope sayi* with late embryos that responded to hatch water from three other brachyurans: *Rhithropanopeus harrisii*, *Uca pugilator*, and *Sesarma cinereum*. Asterisks indicate the first concentration at which the pumping response was significantly different from controls. Control levels for pumping response were established in filtered seawater.

tures first produced significant responses at concentrations corresponding to approximately 100,000 larvae/ml (2.5×10^{-4} *M*) for the *N. sayi* mixture, and approximately 7,000 larvae/ml (8.3×10^{-4} *M*) for the *R. harrisii* mixture. These results suggest that simple mixtures of the amino acids most abundant in pumping factors are not the molecules most active in producing abdomen pumping at the time of larval release.

Discussion

Substances associated with hatching eggs evoked stereotyped larval release behaviors in ovigerous females of the crab *Neopanope sayi*. Responsiveness to these pumping factors varied with embryo age, as did the spontaneous pumping activity of ovigerous crabs. The pumping factors adsorbed to Amberlite XAD-7 resin and could be eluted with methanol, but biological activity did not appear in the methanol eluate until after size fractionation. The presence of small peptides was inferred from the size fractionation and amino acid analysis of a partially purified preparation of hatch water.

For *N. sayi*, a clear variation was observed in the frequency of spontaneous abdomen pumping with embryo age. A possible physiological explanation for this phenomenon is that, as nonliving yolk is converted into embryo, metabolic rate increases, causing increased O_2 demand and waste production. Abdomen pumping by crabs is thought to facilitate O_2 transport to, and waste removal from, developing embryos (Templeman, 1937; Ennis, 1973), hence older embryos with higher metabolic rates would require more water pumped around them.

In contrast to those of *N. sayi*, the spontaneous abdomen pumping rates of *R. harrisii* were independent of

Table I

Effect of passing hatch water through Amberlite XAD-7 resin

Fraction	Test concentration larvae/ml	n	Percentage responding
Hatch water before resin	10	59	69
Hatch water after resin	10	30	33
Control	—	158	23

embryo age (Forward and Lohmann, 1983). This species difference may be due in part to the smaller size of *R. harrisii* egg masses (average size, about 1000 eggs; Forward, unpub. data) compared with *N. sayi* (generally 2000–4000 eggs; De Vries unpub. data). Eggs of the two species are of approximately the same diameter [about 400 μm on the day of hatching (De Vries and Forward, 1990b; De Vries, unpub. data)]. Therefore, the egg masses of *N. sayi* are 2–4 times larger in volume than those of *R. harrisii*, and the former would have a greater total metabolic demand and slower diffusion rate of water through the egg mass. These effects may compound one another, leading to a much greater need for water transport around *N. sayi* eggs, and thus a more pronounced increase in pumping rate as the embryos mature. The increase in egg mass size (De Vries and Forward, 1990b) with embryo age may cause the increase in spontaneous pumping rate by stimulating stretch receptors.

The response of *N. sayi* to hatch water of conspecifics is similar to that observed previously for *R. harrisii* (For-

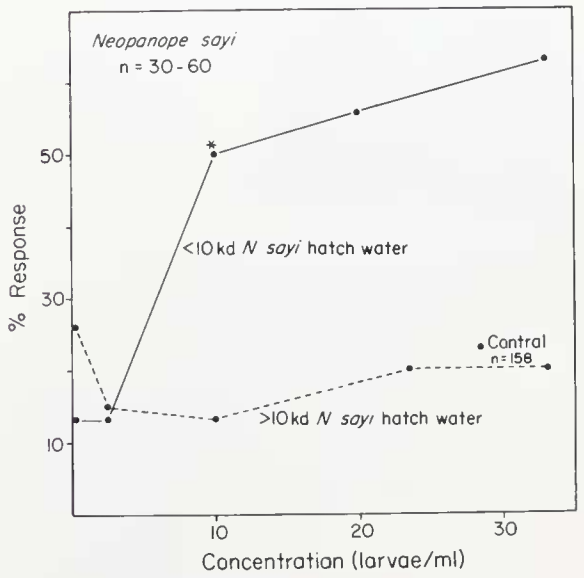

Figure 4. Percentage of ovigerous females of *Neopanope sayi* with late embryos that responded to the retained and non-retained fractions of *N. sayi* hatch water after ultrafiltration with a 10 kDa membrane. The control is for responses in filtered seawater.

Table II

Controls performed for Neopanope sayi *pumping factor experiments*

Solution	Test concentration (larvae/ml)	n	Percentage responding
1. 0.45 μm filtered seawater (FSW) (untreated) (control)	—	158	23
2. 10 kDa fraction MeOH eluate FSW	20	31	23
10 kDa fraction MeOH eluate FSW	50	30	17
3. 1 kDa fraction MeOH eluate FSW	20	30	17
1 kDa fraction MeOH eluate FSW	50	30	17
4. Ovigerous crab essence (OCE) (untreated)	25	25	13
5. 10 kDa fraction MeOH eluate OCE	20	35	26
10 kDa fraction MeOH eluate OCE	50	35	17
6. 1 kDa fraction MeOH eluate OCE	20	35	23
1 kDa fraction MeOH eluate OCE	50	35	6

In all tests, crabs carrying embryos within three days of hatching were used. Test concentrations were calculated as described in the text. Solutions 2–6 produced response rates not significantly greater than control (Solution 1). Ovigerous crab essence was seawater in which ovigerous crabs had been incubated for several hours (details in text).

ward and Lohmann, 1983), which suggests that the model for hatching-time control described for *R. harrisii* also applies for *N. sayi*. In this model, synchronized development of the eggs is believed to result from an unknown interaction between the embryos and the females, but actual hatching time is controlled by the embryos. When the eggs become ready to hatch, several eggs hatch spontaneously, releasing substances into the water that stimulate additional abdomen pumping. This action breaks open the remaining eggs, and the result is the synchronous release of larvae. The release of pheromones by embryos to communicate their readiness to hatch may have adaptive significance. During larval release, female crabs are presumably exposed and therefore at greater risk to predation than at other times (Forward and Lohmann, 1983). In addition, abdominal pumping during larval release is a vigorous, hence an energetically costly activity. Concentration of larval release behaviors to a time when the embryos are ready to hatch may thus decrease the risk of predation and energetic costs to the female.

Substances (pumping factors) that evoke larval release behavior (pumping response) were released at the time of egg hatching and were not released from eggs prior to this time. The effects of different treatments with these pumping factors can be determined by comparing the effective concentrations that evoked a 50% pumping response (EC_{50}; Table IV). When exposed to untreated hatch water, the titer for the EC_{50} was 6 larvae/ml. If this water was passed through a 10 kDa cutoff membrane, the EC_{50} increased to 10 larvae/ml. Because activity could not be detected in the water above the 10 kDa membrane, this decrease in activity did not result from the removal of large active molecules. Two possible explanations are adsorption of active molecules to the membrane during the filtration process, or degradation during the filtration interval by enzymes released by lysed microorganisms during thawing and pressure dialysis of hatch water. Crabs were unresponsive to hatch water that had been passed through the XAD-7 resin, suggesting that active substances

Table III

Amino acid composition of hydrolyzed and unhydrolyzed Neopanope sayi *pumping factor of <1000 daltons*

Amino Acid	Picomolar amount		
	Unhydrolyzed factor	Hydrolyzed factor	Difference
Pro	—	35	35
Cys	53	134	81
Asp	—	13	13
Thr	6	29	23
Ser	15	61	46
Glu	—	47	47
Gly	6	125	119
Ala	6	58	52
Val	28	61	33
Met	3	137	134
Ile	43	123	80
Leu	4	34	30
Tyr	—	—	—
Phe	51	54	3
His	—	—	—
Lys	7	15	8
Arg	—	19	19
Total	222	945	723

Concentrations are calculated for a sample with a titer of 160 larvae/ml.

were removed by the column. The methanol eluate was also inactive. Pressure dialysis of the methanol eluate resulted in a titer of activity comparable to size fractionated (10 kd filtered) hatch water that had not been passed through the resin. This result suggests high recovery of pumping factors from the column, as previously obtained for *R. harrisii* factors (Rittschof *et al.*, 1985).

The apparent absence of activity in the methanol eluate may have resulted because the resin: (1) removed an important component of the pumping factors that was not eluted with methanol; (2) concentrated high molecular weight inhibitory substances in the hatch water; or (3) added inhibitory substances *de novo*. The column did not add inhibitory molecules because XAD-7 resin leaches low molecular weight compounds (Jolley *et al.*, 1981) that would not be removed by size fractionation. Because activity reappeared after passage through the 10 kDa membrane (Fig. 4), the active factors and components were recovered from the resin. Thus, the most likely explanation is that inhibition resulted from concentration of high molecular weight inhibitory substances present in the hatch water.

The EC_{50} for the methanol eluate after passage through the 10 kDa membrane was 13 larvae/ml (Table IV). Because this value is very close to that for untreated hatch water (10 larvae/ml) after passage through the 10 kDa membrane, recovery of active molecules from the XAD-7 column was close to 100%. Rittschof *et al.* (1985) had similar success rates in recovery of active molecules in *R. harrisii* hatch water from an XAD-7 column. The activity of *N. sayi* hatch water remained the same (EC_{50} = 12 larvae/ml) after it had been passed through the 1 kDa membrane, indicating that activity can be attributed solely to molecules that are less than 1 kDa in size.

N. sayi responded to hatch water from *R. harrisii* and *Uca pugilator*, but the EC_{50} values were high at 15 and

Table IV

Effective concentrations for a 50% pumping response (EC_{50}) in crabs carrying late-stage embryos

Hatch water treatment	EC_{50} (larvae/ml)	Data source
Untreated	6	Fig. 2
10 kDa filtered	10	Fig. 4
XAD-7 resin	none	Fig. 2
XAD-7 resin, 10 kDa filtered	13	Fig. 2
XAD-7 resin, 10 kDa, 1 kDa filtered	12	Fig. 2
Rhithropanopeus harrisii hatch water	15	Fig. 3
Uca pugilator hatch water	50	Fig. 3

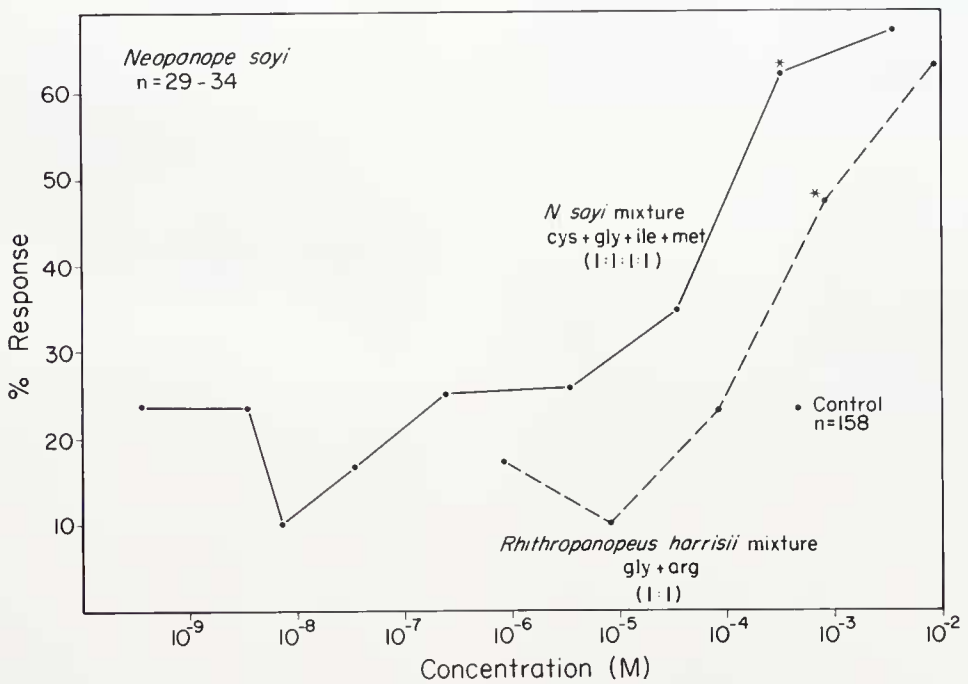

Figure 5. Percentage of ovigerous *Neopanope sayi* with late embryos that responded to different concentrations of mixtures of amino acids. Mixtures represent the main components of hydrolyzed pumping factors of *N. sayi* (equimolar amounts of L-cysteine, glycine, L-isoleucine, and L-methionine) and *Rhithropanopeus harrisii* (equimolar amounts of glycine and L-arginine; Rittschof *et al.*, 1985). Asterisks indicate the first concentration at which the pumping response was significantly different from controls. Control levels for the response were established in filtered seawater.

50 larvae/ml, respectively (Table IV). This result, and the lack of response to *S. cinereum* hatch water, show that cross-reactivity among species occurs, but is incomplete, implying that hatch waters from different species may be similar, but are probably not identical.

Three lines of evidence suggest that the active molecules in hatch water of *R. harrisii* are peptides. First, activity is lost if hatch water is treated with a protease. Second, compositional analysis of partially purified active molecules suggests that they may consist of di- or tripeptides with a neutral amino acid at the amino-terminus and an arginine carboxy-terminus (Rittschof *et al.*, 1985). Third, pure peptides having this structure induce pumping responses (Forward *et al.*, 1987; Rittschof *et al.*, 1989).

Results from the present study support the hypothesis that active substances in hatch water of *N. sayi* are also peptides, though their exact chemical nature is unknown. Evidence for this includes: (1) responsiveness of *N. sayi* to *R. harrisii* hatch water; (2) general similarity in the adsorptive characteristics of *N. sayi* and *R. harrisii* pumping factors toward XAD-7 resin; (3) suggestion of peptides in a partially purified hatch water preparation; and (4) similarity in the response of *N. sayi* and *R. harrisii* to mixtures of amino acids.

The amino acid analysis of *Neopanope sayi* pumping factors indicates that the four main amino acids in the proposed peptides are cysteine, glycine, isoleucine, and methionine. With the exception of glycine, these amino acids have neutral side chains and are hydrophobic. In contrast, the proposed active peptides of *Rhithropanopeus harrisii* contain arginine (present only in low amounts in *N. sayi* factor), which is strongly charged and hydrophilic. Thus the foregoing analysis suggests that the pumping factors of *N. sayi* are more hydrophobic than those of *R. harrisii*.

Bioassays of amino acids suggest that, for both species, simple mixtures of amino acids are not the active components of pumping factors (Rittschof *et al.*, 1985). Mixtures of the amino acids most abundant in the pumping factors produced significant levels of pumping only at concentrations much higher than those present in native hatch waters. The threshold concentrations for responses of *N. sayi* to amino acids were about 10^{-4} *M*, while free amino acid levels based on compositional analysis (Table III) at the threshold concentration of hatch water (2.5 larvae/ml; Fig. 2) were about 3.0×10^{-12} *M* (calculated from Table III). For *N. sayi*, small peptides of undetermined sequence are hypothesized to be the active components of pumping factors as concluded for *R. harrisii* (Rittschof *et al.*, 1985). The source of these peptides is as yet unknown. However we have postulated elsewhere that enzymatic degradation of the egg membranes (De Vries and Forward, 1991b; Rittschof *et al.*, 1990a) produces the pumping factors.

The chemical mediation of a diversity of behaviors has been described in virtually all phyla and may be particularly important in aquatic environments (*e.g.*, Knight-Jones, 1953; Collins, 1975; Trott and Dimock, 1978; Derby and Atema, 1980; Tierney and Dunham, 1982; Rittschof *et al.*, 1983, 1984). In particular, proteins and peptides elicit behaviors in various taxa, including feeding behavior in the snail *Ilyanassa* (=*Nassarius*) *obsoleta* (Carr *et al.*, 1974), creeping in predatory snails (Rittschof *et al.*, 1984), and metamorphosis in the sand dollar *Dendraster excentricus* (Burke, 1984) and the abalone *Haliotis* (Morse, 1988). Among crustaceans, serine protease generated peptides are implicated in hermit crab shell acquisition behavior (Rittschof, 1980; Lepore and Gilchrist, 1988; Rittschof *et al.*, 1990b), barnacle attachment behavior (Rittschof, 1985) and metamorphosis (Tegtmeyer and Rittschof, 1989), and crustacean larval release (Rittschof *et al.*, 1990a). Rittschof and Bonaventura (1986) argue that distinct advantages are inherent in the use of peptides as chemical cues in aquatic systems, including: (1) increased complexity of primary structure (compared to amino acids), allowing opportunity for increased response specificity; (2) background concentrations in marine systems are low (Mopper and Lindroth, 1982), allowing for high signal to noise ratios; and (3) metabolic inexpensiveness, because they need not be synthesized *de novo*, but may be broken down from existing structural and metabolic components.

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